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Merocyanine 540 as a fluorescence indicator for molecular packing stress at the onset of lamellar-hexagonal transition of phosphatidylethanolamine bilayers

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Abstract

The fluorescence of Merocyanine 540 (MC 540) is sensitive to the molecular packing of membrane lipids. Therefore, the fluorescence of MC 540 is expected to be sensitive to the curvature-related packing stress at the onset of the lamellar-hexagonal phase transition. We measured the fluorescence intensity of MC 540 when the temperatures of lipid bilayers approached their lamellar-hexagonal phase transitions. The fluorescence of MC 540 in the presence of egg and dioleoylphosphatidylethanolamine bilayers increased at the respective lamellar-hexagonal phase transitions of these lipids. Furthermore, increases in fluorescence intensity were also observed at temperatures just below their phase transitions. The enhanced fluorescence was not due to the specific interaction of the dye with the ethanolamine headgroup, because no such increase was observed when the probe was exposed to phosphatidylethanolamines which do not form hexagonal phase within the range of applied temperature. In addition, when the temperature of the lamellar-hexagonal phase transition was shifted, by the addition of a small amount of phosphatidylcholine, the dependence of the fluorescence intensity on temperature was modified accordingly. We postulate that the change of MC 540 fluorescence intensity at temperatures approaching the lamellar-hexagonal phase transition reflects changes in the partition of MC 540 into the fluid lipid phase. The change in partition is influenced by the curvature stress in bilayers at temperatures just below the lamellar-hexagonal phase transition.

Keywords: Merocyanine; Phosphatidylethanolamine; Membrane; Lipid bilayer; Lamellar-hexagonal phase transition

1. Introduction

Biological membranes commonly contain lipids which alone do not self-assemble in a bilayer form under physiological conditions. These lipids, when dispersed in water, form high curvature structures such as the inverted cubic phase or the inverted hexagonal phase [1,2]. Certain biological membranes contain a large portion of these 'unstable' lipids. In addition, the lipid composition of this 'unstable' fraction varies from membrane to membrane. It has been postulated that the physical properties of these lipids, in addition to their chemical nature, are important factors in fulfilling their physiological functions [3,4].

There have been many studies of the lamellar-hexagonal phase transition of these lipids [3–5]. It has been argued that the spontaneous shape of a lipid

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molecule in an interfacial environment plays an important role in determining the lamellar-hexagonal phase transition. Lipids with a small volume of the headgroup, as compared to the volume of the hydrophobic part, are more prone to form inverted hexagonal or cubic phases [6]. A large body of experimental data was given to support this model [3,7,8]. The gradual buildup of curvature stress near the lamellar-hexagonal phase transition is expected to produce strain and/or structural defects [4,8]. The buildup of membrane stress prior the lamellar-hexagonal phase transition modifies many enzymatic activities [3,9–11]. Changes caused by stress in the lipid bilayer might also alter the membrane morphology and increased ion permeability [12].

To study the putative pre-transitional packing stress, we apply a fluorescence probe, Merocyanine 540 (MC 540) which has well-defined fluorescence properties. The fluorescence of MC 540 depends on the polarity of the environment [13]; therefore, the partition of the dye into a lipid bilayer is easily detectable. When the probe is in the lipid environment, its emission maximum is red-shifted and the quantum efficiency enhanced. Several experiments have shown that the surface binding and fluorescence of MC 540 depend on the molecular packing of the lipid bilayer. Lelkes and Miller showed that the optical density of MC 540 increases in the presence of lipid vesicles at the fluid-gel phase transition [14,15]. Later, Williamson and coworkers observed that the fluorescence of MC 540 increases in the presence of fluid and loosely packed membranes when compared to that in water and in the presence of lipid vesicles in the gel phase [16]. MC 540 has been used to report changes of molecular packing upon the addition of cholesterol into phospholipid bilayers, as well as in bilayers under osmotic tension [17]. These findings are confirmed by studies on monolayers where the molecular packing is correlated to the fluorescence of MC 540 [18]. The effect of the molecular packing on the fluorescence of MC 540 is best illustrated when the membrane experiences the gel-fluid phase transition, where the fluorescence of the dye increases in the step-like fashion [19]. The absence of a fluorescence maximum at the main phase transition suggests that the fluorescence of MC 540 is not significantly influenced by domain boundaries in the lipid bilayer. In this paper we used MC 540 as a probe of molecular packing in phosphatidylethanolamine membranes approaching the lamellar-hexagonal phase transition.

2. Materials and methods

2.1. Chemicals

Phosphatidylethanolamine, transesterified from egg phosphatidylcholine (TPE), dioleoylphosphatidylethanolamine (DOPE) and dioleoylphosphatidylcholine (DOPC) were obtained from Avanti Polar Lipids (Alabaster, AL). 1-Palmitoyl,2-caprioylphosphatidylethanolamine was a gift from Dr. Ching-Hsien Huang (University of Virginia). MC 540 and *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)dipalmitoyl-L-α-phosphatidylethanolamine (NBD-PE) were purchased from Molecular Probes (Eugene, OR). Sodium dithionite and other chemicals were from Sigma (St. Louis, MO).

2.2. Preparation of vesicles

Lipids mixed in chloroform were evaporated to dryness, resuspended in the 0.1 M KCl, 4 mM HIS/TES buffer at pH 7.4, and vortexed. NBD-PE was added to lipids in chloroform before evaporation. The vesicle suspension was passed ten times through a 0.2 μm cellulose filter (Costar, CA) to obtain a uniform size vesicle population. The size of vesicles was determined using the dynamic light scattering method (Submicron Particle Sizer, Model 370, NICOMP, Santa Barbara, CA). Vesicle sizes vary from 0.175 μm to 0.198 μm according to composition, and standard deviation never exceeded 0.007 μm. All steps were performed on ice to prevent the formation of the hexagonal phase. A small volume of vesicles from the stock solution was added to the preincubated cuvette with buffer solution before the fluorescence was measured.

2.3. The fluorescence of MC 540

The fluorescence intensity was measured on an SLM 8000 fluorimeter with an excitation and an emission wavelength at 540 nm and 585 nm respectively. MC 540 was added to the vesicle suspension

from the ethanol stock solution. The ethanol concentration in the sample never exceeded 0.5% by volume. The fluorescence was measured after its intensity stabilized. The fluorescence intensity was measured for at least five MC 540 concentrations. The concentration of MC 540 was within the range of linear relation between fluorescence intensity and the concentration of the dye. The increase of fluorescence with the concentration of MC 540 was calculated using the least squares method. All data presented in this paper are slopes of a fluorescence increment versus dye concentration. The concentration of MC 540 in the sample never exceeded 0.1 μg/ml. Data used in calculations have been corrected for inner filter effect and light scattering [20]. Calculated corrections never exceeded 5% of measured values.

The correction for the temperature effect on the fluorescence of the dye was applied according to the procedure described previously [19]. In short, the fluorescence of MC 540 was measured in ethanol within the applicable temperature range and used as a reference curve. Then the fluorescence of MC 540 in the presence of lipids was divided by the fluorescence intensity obtained from the reference curve.

2.4. Measurements of the membrane permeability

As we have shown previously, defects in the lipid bilayer facilitate the penetration of dithionite through the hydrophobic core of the membrane [12]. Consequently, the slow decrease of the fluorescence of NBD-PE after addition of dithionite can be used as a measure of defects in the lipid bilayer. A small volume of dithionite (30 μl) was added to vesicle suspension (3 ml) from 1 M stock solution [12,21]. The fluorescence intensity of NBD-PE was measured continuously before and after the addition of dithionite (excitation and emission wavelengths were 465 nm and 535 nm respectively). The fluorescence intensity was normalized to its initial value. Slopes presented in Fig. 6 were calculated from the plot of the logarithm of the fluorescence intensity versus time using the least squares method.

2.5. X-Ray diffraction

Small angle X-ray diffraction was used to determine the phase transition of lipid samples [22]. Lipid samples in 1.5 mm diameter thin-wall glass tubes (Charles Supper, Natick, MA) were placed in a thermostat holder of a Frank-type camera. Small angle diffraction patterns were collected by a position sensitive wire detector (Tennelec, Oak Ridge, TN) at various temperatures. The X-ray beam was generated by a Rigaku RU-200B rotating anode generator.

3. Results

As shown previously [12–17], the fluorescence intensity of MC 540 in the presence of fluid and loosely packed membranes is substantially higher then that in the presence of well-organized (gel phase) bilayers. We made use of the dye sensitivity to lipid packing, to measure the changes in a bilayer as it approaches the lamellar-hexagonal phase transition.

The dependence of the fluorescence intensity of MC 540 on temperature, in the presence of TPE vesicles, is shown in Fig. 1. The lamellar-hexagonal phase transition for TPE was listed to be at 55–65°C [23]. When TPE vesicles are incubated in the buffer at pH 7.4 the fluorescence intensity of MC 540

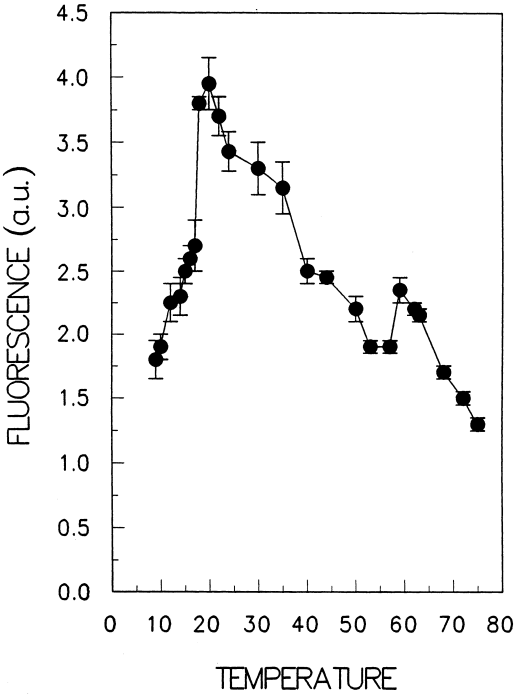

Fig. 1. Fluorescence intensity of MC 540 in the presence of egg-PE vesicles as a function of temperature.

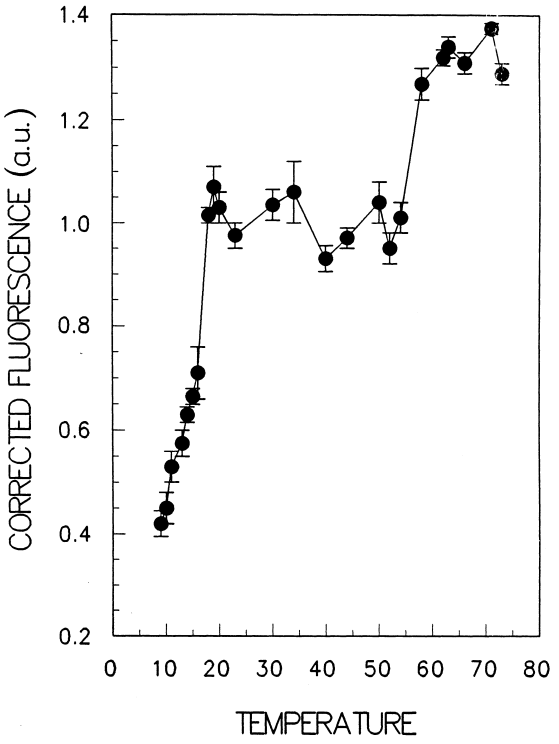

Fig. 2. Fluorescence intensity of MC 540 in the presence of egg-PE vesicles (Fig. 1) after correction for the temperature effect as described in Section 2.

reaches a maximum at about 20°C. Above that temperature the fluorescence falls continuously until it increases again at about 60°C. The temperature of the second increase coincides with the lamellar-hexagonal phase transition, as determined using X-ray diffraction measurements (60–65°C). The fluorescence and X-ray diffraction were measured using the same batch of TPE.

We have shown previously that the fluorescence efficiency of MC 540 depends on temperature, and decreases when temperature rises [19]. Therefore, the fluorescence of the dye shown in Fig. 1 is affected by both: the temperature and the changing lipid molecular packing. To separate these two effects, the fluorescence intensity of MC 540 was corrected for temperature as described previously [19]. The corrected fluorescence intensity of MC 540 in the presence of TPE vesicles is shown in Fig. 2. The intensity increases continuously below 20°C. Above 20°C the fluorescence intensity remains constant until the temperature of the lamellar-hexagonal phase transition is reached when the fluorescence rises again.

The enhanced fluorescence of MC 540 suggests decreased organization of lipids [16] and/or increased membrane surface area available for the binding of the dye. The gel-fluid phase transition temperature of TPE is well below 0°C. Therefore, this increase is not related to the gel-fluid phase transition. Data in Fig. 2 indicate that TPE bilayers become increasingly 'fluid' until 20°C. Above this temperature, no further changes are detected. The changes below the onset of the lamellar-hexagonal phase transition are believed to be a result of the buildup of curvature stress within the lipid bilayer [4]. When the temperature reaches the lamellar-hexagonal phase transition, the fluorescence increases again in a step-like fashion, indicating that there is a major molecular reorganization.

The elevated level of stress within the lipid matrix at the onset of the lamellar-hexagonal phase transition is likely to cause the formation of defects and may increase the permeability to ions through the lipid bilayer. Fig. 3 shows the temperature dependence of permeability of TPE vesicles to dithionite ions. The permeability is given as the decrease of

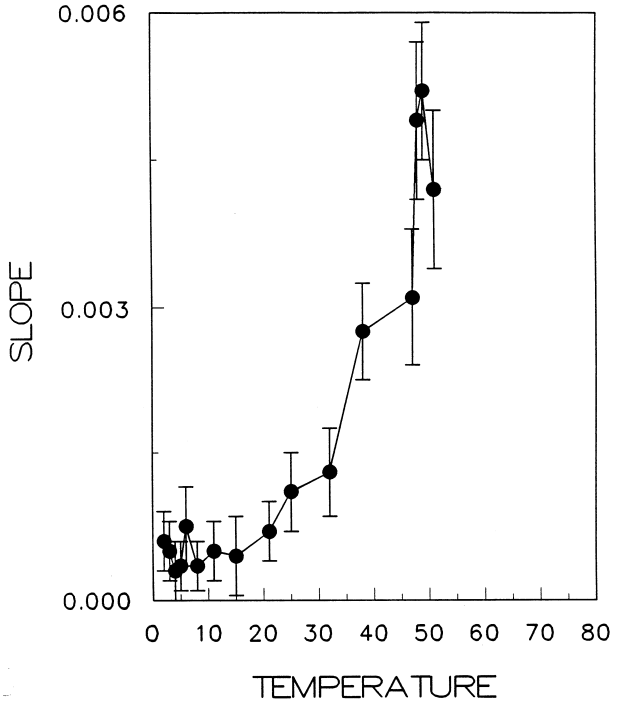

Fig. 3. Permeability of egg-PE to dithionite as a function of temperature. The permeability is measured as a slope of the logarithm of the fluorescence intensity of NBD-PE versus time.

fluorescence intensity of NBD-PE at the inner surface of PE vesicles, due to the irreversible degradation of NBD by dithionite ions that have passed through the vesicle walls [12]. The permeability increases with temperature and reaches a maximum near the lamellar-hexagonal phase transition. Above the phase transition, open hexagonal structures are accessible to the dithionite from the aqueous phase and permeability measurements are meaningless. This result shows that the number of defects acting as permeation sites in the lipid bilayer is large when the temperature is close to that of the lamellar-hexagonal phase transition.

Fig. 4 shows the corrected fluorescence of MC 540 in the presence of DOPE vesicles. This phosphatidylethanolamine has the lamellar-hexagonal phase transition at about 10°C [23] whereas the gel-fluid phase transition temperature is again below 0°C. The changes of the fluorescence intensity are similar in character to that obtained in the presence of TPE vesicles. The fluorescence increases until about 7°C, stabilizes and rises again at the temperature of the lamellar-hexagonal phase transition (10°C).

When vesicles are formed from the mixture of these unsaturated phosphatidylethanolamines with

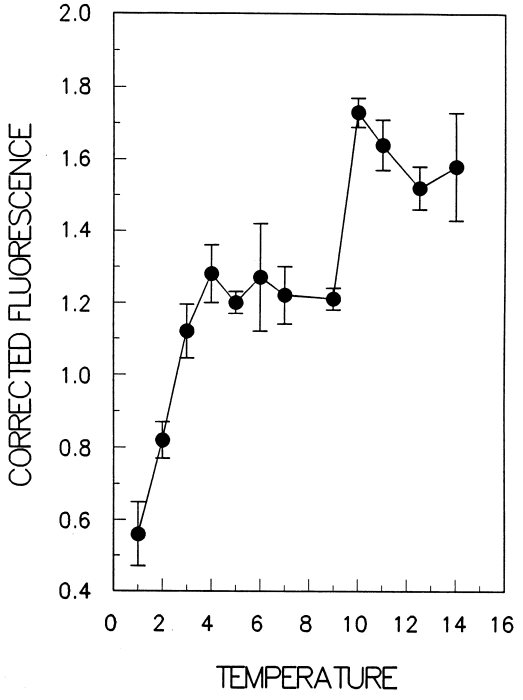

Fig. 4. Temperature-corrected fluorescence intensity of MC 540 in the presence of DOPE vesicles.

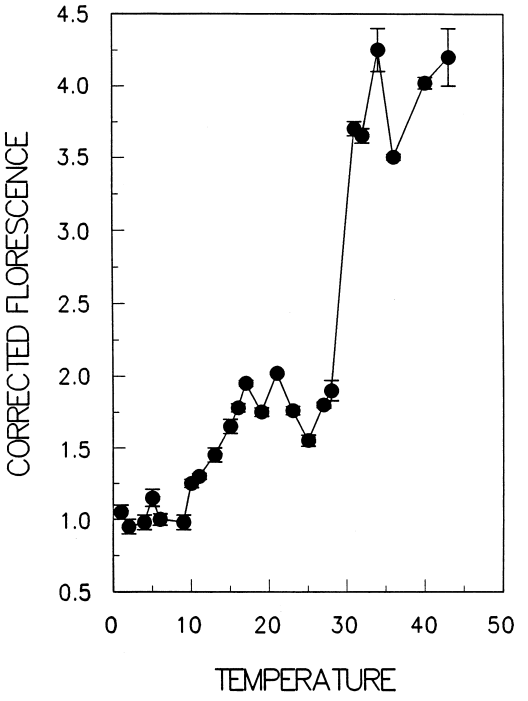

Fig. 5. Temperature-corrected fluorescence intensity of MC 540 in the presence of vesicles formed from the mixture of DOPE and 5 mol% DOPC.

lipids that 'stabilize' the lamellar structure, the temperature of the lamellar-hexagonal phase transition is expected to rise [7]. We studied the fluorescence of MC 540 in the presence of vesicles formed from the mixture of DOPE with 5 mol% DOPC (Fig. 5). Only a single increase of the fluorescence of MC 540 is observed within the temperature range from 10°C to 30°C. Measurements of the fluorescence intensity at higher temperatures (>50°C), are not practical due to the rapid decrease of the quantum efficiency of the dye.

Finally the fluorescence of MC 540 in the presence of vesicles formed from 1-palmitoyl,2-caprioyl-phosphatidylethanolamine PE(C10,C18) was measured. This phosphatidylethanolamine does not experience a lamellar-hexagonal phase transition within the applied temperature range. The fluorescence intensity decreases with temperature in a way that is similar to that observed for phosphatidylcholine membranes [19]. The temperature corrected fluorescence intensity of this PE is shown in Fig. 6. There is no change in the corrected fluorescence throughout the whole range of temperatures.

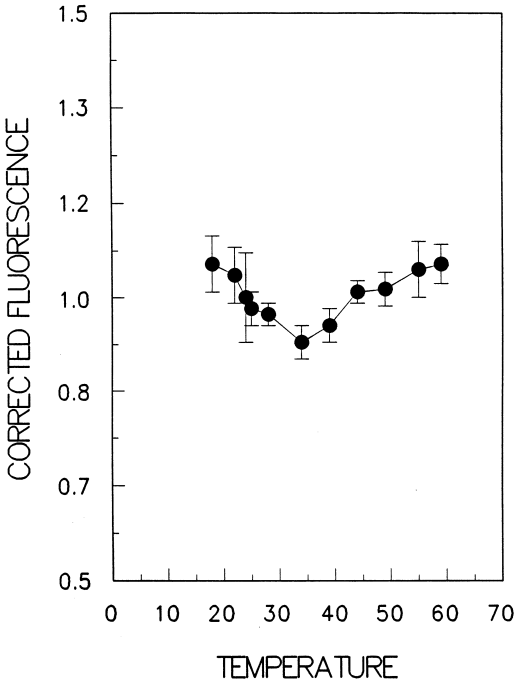

Fig. 6. Temperature-corrected fluorescence intensity of MC 540 in the presence of C16,C10-PE vesicles as a function of temperature.

4. Discussion

The interaction of MC 540 with lipid membranes has been a subject of extensive studies. Initially MC 540 was used as a potential sensitive dye [24]. Later it was established that fluorescence intensity of the dye depends also on the molecular packing of lipids in bilayers [16]. For example, during the main phase transition, the rise of the fluorescence reflects changes in the molecular packing of lipids [16,19]. It has been established that MC 540 binds to the bilayer surface and does not penetrate deeper than the glycerol level [14,15,25,26]. Consequently, the probe may be used as a molecular packing reporter located near the lipid-water interface. We have shown that the increased fluorescence of the dye in the presence of vesicles above their main phase transition reflects enhanced partitioning of MC 540 into the fluid bilayer [19]. The enhanced partitioning is not a result of changes in the vesicle morphology. During the main phase transition, the continuity of the bilayer is preserved and the total amount of the membrane surface area accessible to the fluorophore does not change as much as that during the lamellar-hexagonal transition.

The lamellar-hexagonal transition is determined by the curvature stress in monolayer leaflets of a bilayer. Curvature stress builds up when the lipid molecules are packed in an assembly form that is different from their lowest energy form of assembly (i.e., spontaneous curvature) [8]. A considerable percentage of cell membrane lipids, such as unsaturated PE, have non-planar spontaneous curvatures at physiological temperature [4]. Raising the temperature in a monolayer leaflet containing such lipids would increase the curvature stress, and would eventually reach the energy threshold for the lamellar-hexagonal transition. At temperatures just below the transition, the curvature stress of the bilayer is near its maximum, and the bilayer is unstable. Several biomembrane functions have been related to the curvature stress in their lipid bilayers [4,27]. This is the state which is said to be preferred by many organisms for their optimal membrane activities [28]. Detecting this pre-transition packing stress is, therefore, more important than detecting the transition itself.

Changes in the molecular packing of lipids in the bilayer at and prior to the lamellar-hexagonal phase transition have been measured previously in a number of studies using a variety of techniques. For example, Hong and coworkers have shown that the fluorescence of *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine (NBD-PE) increases at the lamellar-hexagonal phase transition [29]. The fluorescence moiety of NBD-PE is located at the membrane surface. Therefore, the enhanced fluorescence reflects changes taking place at the water-lipid interface such as the hydration of the lipid or the degree of hydrogen bonding of the headgroup.

Changes in the properties of bilayers prior to the lamellar-hexagonal phase transition were measured by Epand and Leon [30]. They showed that the fluorescence of *N*-densyl-L-Lys located in the lipid bilayer changes with the approaching of hexagonal phase transition. A similar effect was observed with other fluorescent probe, laurdan (6-dodecanoyl-2-(dimethylamino)naphthalene). Both probes are sensitive to changes taking place close to the water-lipid interface.

The increase of fluorescence intensity of MC 540 at the lamellar-hexagonal phase transition indicates a

drastic change in the membrane morphology. This increase was observed in the presence of egg-PE or DOPE vesicles at temperatures where the respective phase transition occurred. The rise of the fluorescence intensity of MC 540 at the lamellar-hexagonal phase transition is likely to be caused by topological changes and/or alterations of molecular packing at the membrane surface. The formation of the hexagonal phase would increase the number of lipid headgroups able to interact with MC 540 from the aqueous phase. Changes of the surface properties caused by the modification of molecular packing are also expected at the lamellar-hexagonal phase transition [31].

More interestingly, the corrected fluorescence of MC 540 at temperatures *below* the lamellar-hexagonal phase transition was also found to increase with temperature, up to a point below the lamellar-hexagonal transition. This alteration of the fluorescence is likely to be a result of changes in the dye binding to the bilayer surface as a consequence of the building-up of curvature stress. We did not observe any traces of hexagonal phase structure at these temperature ranges by X-ray diffraction, nor was there any observation by electron microscopy at temperatures that the fluorescence of MC 540 increased with temperature. Therefore, the basic bilayer structure had not been compromised, and the bilayer surface exposed to the dye in the aqueous phase was not expected to change significantly. The rising fluorescence is believed to indicate the decrease of molecular packing order, to the extent that affects the dye binding to the bilayer surface. The changes in the lipid molecular packing only occur at temperatures not far below the lamellar-hexagonal phase transition. The bilayer formed from PE(C18;C10) and phosphatidylcholine [19] did not show the enhanced binding of MC 540.

Alterations of the bilayer properties, such as permeability or the presence of defects, at temperatures just below the lamellar-hexagonal phase transition, are caused by changes in the molecular packing lipid bilayer, and culminate in the formation of the reverse hexagonal phase. Increasing membrane permeability to dithionite indicates that defects similar to these at the domain boundary [12] are formed throughout the lipid bilayer. As the temperature approaches the lamellar-hexagonal transition, morphological changes in lipids in the form of non-bilayer structures have been observed. Small lipidic particles form in phosphatidylethanolamine lipid bilayers at temperatures preceding the lamellar-hexagonal phase transition [22,32–34]. As these non-bilayer structures form, the curvature stress relaxes, resulting in the cessation of the increase in fluorescence intensity with temperature just below the lamellar-hexagonal transition. Our knowledge of the nature of interaction between MC 540 and lipids suggests that such fluorescence changes are caused by the modification of the dye-bilayer interaction in the headgroup region of the bilayer. Epand and Leon [30] have made similar observations.

In summary, MC 540 reports the lamellar-hexagonal phase transition in the same manner as the gel-fluid phase transition. In addition, with fluid bilayers, the fluorescence of the dye changes at temperatures below the lamellar-hexagonal transition, indicating the building up of curvature stress. It provides a convenient way to detect this elusive membrane property.

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